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Molecular characterization of *Moniezia sichuanensis* in captive musk deer (*Moschus berezovskii*)

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Abstract

The musk deer (*Moschus berezovskii*) is an economically important species from which musk is extracted and used in perfumes and medicines. Cestodes (parasitic flatworms) of the genus *Moniezia* are important parasites that infect this endangered species and can cause high mortality in young deer. In 1982, *Moniezia (S.) sichuanensis* sp. nov. was described from a specimen obtained from wild musk deer. The new species was distinct from the other described species of *Moniezia* by the sawtooth-shaped interproglottidal glands, the thick vagina and the absence of a cirrus spine. In the present study, 12 cestodes collected from musk deer were examined morphologically and confirmed to be *M. sichuanensis*. Molecular characterization was performed by amplifying and comparing the internal transcribed spacer 1 (ITS1) and 5.8S rRNA gene (ITS1–5.8S) of ribosomal DNA with available sequences from other *Moniezia* species. The amplified sequences ranged from 761 to 764 bp and similarity ranged from 98.7–100%, compared to 67.8–92.4% with other *Moniezia* spp. Construction of a phylogenetic tree using the neighbour-joining method indicated that all 12 ITS1–5.8S sequences formed a single clade, confirming *M. sichuanensis* as a separate species. This study provides novel molecular insight into *M. sichuanensis* that could prove useful for future diagnosis and control of monieziasis in musk deer.

Introduction

The musk deer (*Moschus berezovskii*), which is unique to Asia, is both rare and medicinally useful. It is categorized as endangered in the IUCN Red List (Wang & Harris, 2008) and is a Class I nationally protected wildlife species (Smith *et al*., 2010). It is also an economically important species due to its musk, harvested from the scent gland of mature males (Green, 1986), which is used in perfumes as well as in around 300 traditional Chinese medicine prescriptions (Sheng & Liu, 2007). Due to the high value of musk, a programme of artificial rearing began in China in the late 1950s. Techniques for obtaining musk without harming the animals were developed to ensure conservation of the deer and the sustainable use of musk. To date, more than 10,000 musk deer have been raised in various provinces, including Sichuan and Shaanxi, and these two provinces account for the majority of musk deer (Wang *et al*., 2010a; Li & Jiang, 2014; Cai *et al*., 2016).

Cestodes of the genus *Moniezia* in the family Anoplocephalidae are an important parasite of

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herbivorous ruminant animals, including musk deer, causing monieziasis in domestic and wild animals, especially juveniles. These parasites have a worldwide distribution and are present in 30 provinces in China, including Inner Mongolia, Xinjiang, Qinghai, Guizhou, Sichuan and Tibet (Lin & He, 1975; Shen, 2005; Yang & Zhang, 2013). Monieziasis poses a great threat to artificial breeding programmes by causing high mortality of young deer (Yang *et al.*, 2002). In 1982, a tapeworm found in wild musk deer in Barkam, Sichuan Province, was defined as the new species *Moniezia (S.) sichuanensis* sp. nov. in a new subgenus (*Sinicamoniezia* subgen. nov.) of *Moniezia*, based on the unusual sawtooth-shaped interproglottidal glands situated at the anterior part of each proglottid, the thick vagina and the absence of a cirrus spine (Wu, 1982). Subsequent research has focused on the morphological characteristics, biology and epidemiology of *M. sichuanensis*, as well as controlling monieziasis induced by this species in captive musk deer (Yang *et al.*, 2001a, b). However, genetic information for the genus *Moniezia* is of limited availability, making it difficult to explore the molecular characteristics of this species. Genetic information has been reported recently for *M. expansa*, *M. benedeni* and *M. monardi* (Ohtori *et al.*, 2015), as well as two undefined *Moniezia* species (Wickström *et al.*, 2005), making it possible to study *M. sichuanensis*.

In this study, 12 *M. sichuanensis* specimens were collected from musk deer in five distinct areas. To better understand the molecular characteristics of *M. sichuanensis*, the internal transcribed spacer 1 (ITS1) and 5.8S rRNA gene (ITS1–5.8S) of ribosomal DNA were amplified and analysed.

Materials and methods

Tapeworm collection and staining

Twelve tapeworms were isolated from newly retrieved faeces of 12 musk deer after conventional deworming using praziquantel tablets (25 mg/kg body weight, single oral administration). Tapeworms were placed immediately in disposable plastic bags, labelled and shipped to Sichuan Agricultural University. A single *M. expansa* tapeworm from a goat was provided by the Department of Parasitology, Sichuan Agricultural University. Specific information about the tapeworm samples is shown in table 1 and fig. 1. Each individual cestode was washed in saline and divided into two parts (mature and immature). Mature segments were fixed in 70% alcohol and stained with carmine for morphological examination of the interproglottid region and reproductive organs (Wu, 1982; Yang *et al.*, 2001b; Yang & Zhang, 2013). Immature segments were frozen and stored at −70°C for DNA extraction.

DNA extraction, PCR amplification and amplicon sequencing

Total DNA was extracted from immature segments of the 13 samples (12 from musk deer and one from goat) using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers ITS1-F (5′-GCTGCTACCCGCATGATGTT-3′) and ITS1-R (5′-GGCAAGCCTATAGCCGCAAT-3′) (Nguyen *et al.*, 2012) were used to amplify the ITS1–5.8S region of ribosomal DNA. Polymerase chain reaction (PCR) was performed in a 25-µl reaction volume containing 12.5 µl of *Taq* PCR Master Mix (Tiangen Biochemical Technology Co., Ltd., Beijing, China), 8.5 µl of double-distilled water (ddH_2O), 2 µl of DNA template and 1 µl of each forward and reverse primer (working concentration = 10 pmol/l). Reaction cycles consisted of an initial denaturation step at 94°C for 5 min; followed by 30 cycles of 94°C for 45 s, 56°C for 30 s and 72°C for 45 s; with a final extension of 72°C for 10 min. PCR products were visualized by 1% agarose gel electrophoresis using GoldenView staining. Amplicons of expected size were purified and cloned into the pMD19-T vector and introduced into *Escherichia coli* (DH5α). To ensure maximum accuracy, three positive colonies of each sample were sent for commercial sequencing (Invitrogen, Shanghai, China). Consensus sequences were utilized for subsequent analyses.

Table 1. Species information and GenBank accession numbers of the ITS1–5.8S sequences for *Moniezia* spp.

Parasite sample	Locality	Host	GenBank accession no.
M. sichuanensis/MEK01	Barkam (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377887
M. sichuanensis/MEK02	Barkam (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377888
M. sichuanensis/MEK03	Barkam (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377889
M. sichuanensis/BX01	Baoxing (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377883
M. sichuanensis/BX02	Baoxing (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377884
M. sichuanensis/BX03	Baoxing (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377885
M. sichuanensis/BX04	Baoxing (Sichuan, China)	Musk deer (*M. berezovskii*)	KX377886
M. sichuanensis/ZP01	Zhenping (Shaanxi, China)	Musk deer (*M. berezovskii*)	KX377882
M. sichuanensis/FX01	Fengxian (Shaanxi, China)	Musk deer (*M. berezovskii*)	KX377878
M. sichuanensis/FX02	Fengxian (Shaanxi, China)	Musk deer (*M. berezovskii*)	KX377879
M. sichuanensis/LB01	Liuba (Shaanxi, China)	Musk deer (*M. berezovskii*)	KX377880
M. sichuanensis/LB02	Liuba (Shaanxi, China)	Musk deer (*M. berezovskii*)	KX377881
M. expansa	Sichuan, China	Goat	KX377890
M. monardi	Japan	Japanese serow	AB_367791
M. expansa	Japan	Sheep	AB_367793
M. benedeni	Japan	Cattle	AB_367792
Moniezia sp. B1	Poland	*Bison bonasus*	EF_606904
Moniezia sp. LMW-2004	Finland	*Rangifer tarandus*	AY_752651

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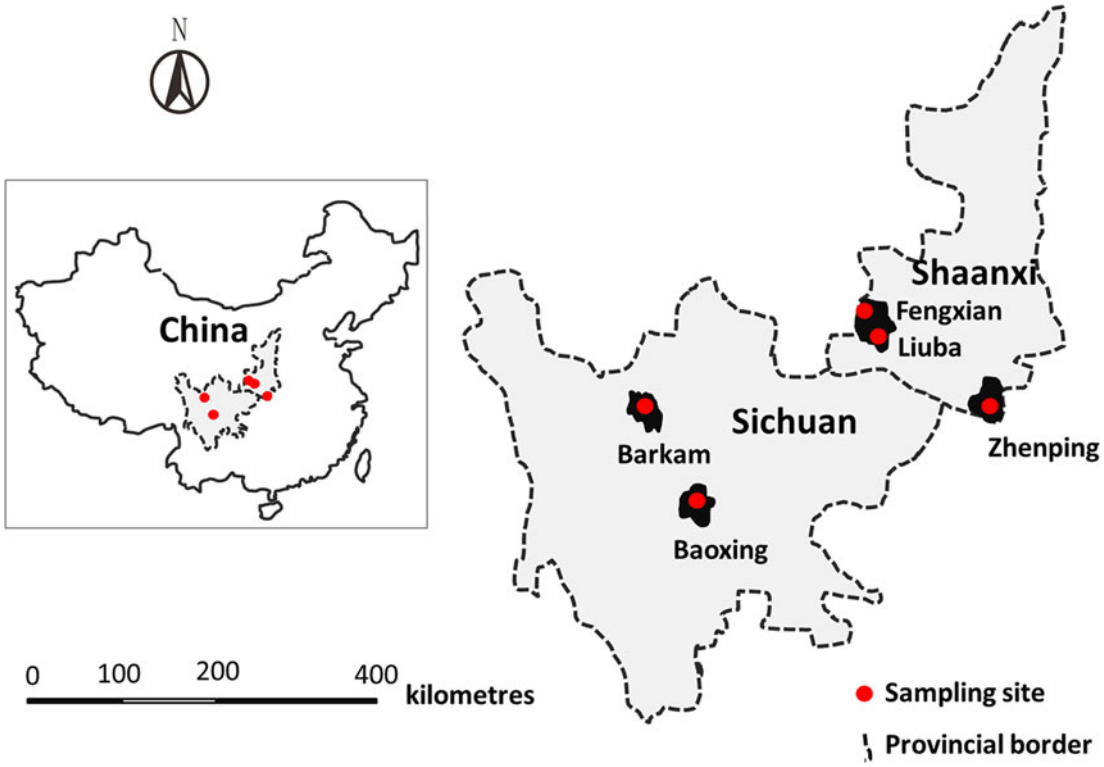

Fig. 1. Sampling sites from which the 12 *M. sichuanensis* specimens were collected from musk deer raised in Sichuan and Shaanxi provinces, China.

Phylogenetic analysis

The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used to compare the obtained nucleotide sequences with published gene sequences from other *Moniezia* spp. deposited in the National Center for Biotechnology Information (NCBI). The corresponding sequences are listed in table 1. Multiple sequence alignment was performed using DNAMAN software package, 2005 (Lynnon Corporation, San Ramon, California, USA) and the genetic similarities between species were also determined using this software. Phylogenetic trees of relevant species were constructed by the neighbour-joining method using MEGA 5.0 (http://www.megasoftware.net) based on genetic distances calculated by the Kimura 2-parameter model. The robustness of the tree was tested by bootstrapping with 1000 replicates. Phylogenetic analysis was performed using sequences generated from PCR analysis of the ~760 bp ITS1–5.8S fragment along with sequences obtained from NCBI (table 1). *Dipylidium caninum* (GenBank accession number: AM_491338) was used as the outgroup.

Results

The interproglottidal glands from all 12 specimens from musk deer had a clear sawtooth shape, the cirrus spine was absent and the vagina was thick. Based on previously published work (Wu, 1982) (fig. 2), all 12 specimens from the five areas were identified as *M. sichuanensis* via morphological analysis. A 761–764 bp ITS1–5.8S fragment and a 754 bp ITS1–5.8S fragment were successfully amplified. All sequence information has been deposited in GenBank under accession numbers KX377878–KX377890.

Multiple alignment showed that ITS1–5.8S sequences amplified from the 12 *M. sichuanensis* specimens shared between 98.7 and 100% similarity, and 67.8–92.4% similarity with other *Moniezia* spp. Similarity among the seven samples from Sichuan province was between 99.2 and 100%, and similarity was 99.6–100% among the five samples from Shaanxi province. As shown in table 2, nucleotide indels and substitutions were observed in the ITS1–5.8S sequences of the 12 *M. sichuanensis* specimens. According to the phylogenetic tree based on ITS1–5.8S sequences (fig. 3), the 12 *M. sichuanensis* samples clustered together (98.7–100% similarity), and also with *M. monardi* (92% similarity), *M. expansa* (77% similarity), *M. benedeni* (81% similarity), and two other unnamed *Moniezia* cestodes (77% and 74% similarity) in another branch. Based on the morphological observations and phylogenetic analyses, *M. sichuanensis* was confirmed as a separate species distinct from known species.

Discussion

Defining species within the genus *Moniezia* has proved controversial, and in an attempt to simplify the systematics of this genus, it was divided into three subgenera

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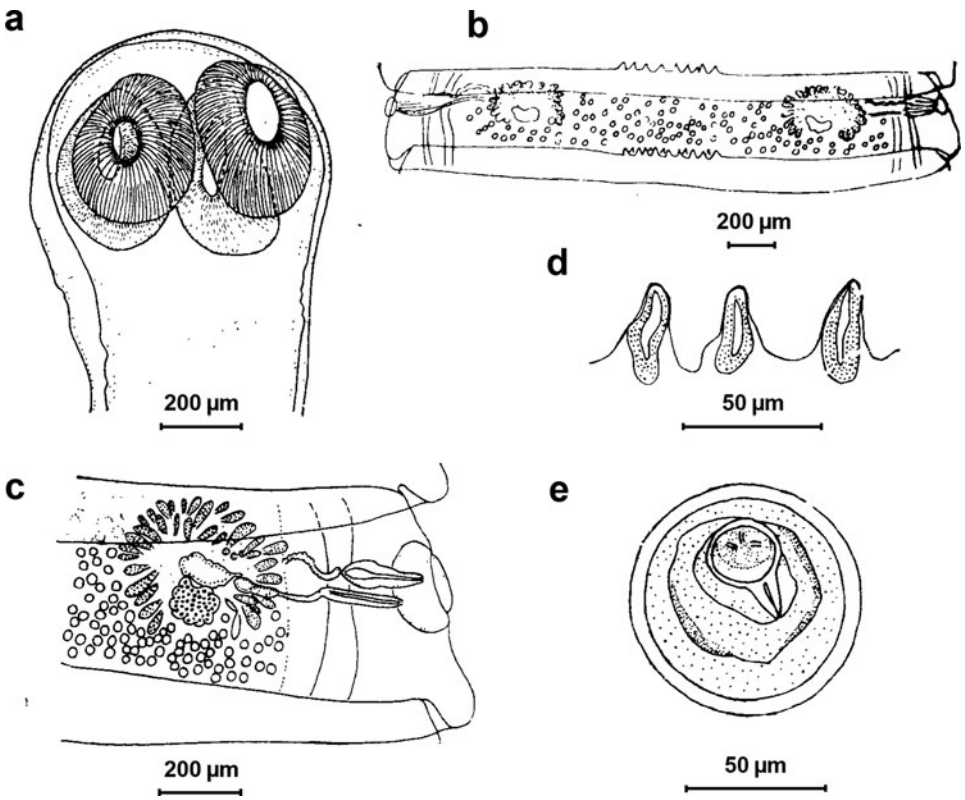

Fig. 2. Line drawings of *Moniezia (S.) sichuanensis* sp. nov.: (a) scolex; (b) mature proglottid; (c) mature proglottid (reproductive organs magnified); (d) interproglottidal glands; and (e) eggs. Drawings and interpretation are based on previously published work (Wu, 1982).

based on the presence or absence and shape of interproglottidal glands: (1) *M. baeriezia* (interproglottidal glands absent), including *M. mettami*, *M. rugosa* and *M. baeri*; (2) *M. moniezia* (rosette-shaped interproglottidal glands) including *M. expansa* and *M. monardi*; (3) *Moniezia blanchariezia* (line-shaped interproglottidal glands) including *M. benedeni* and *M. pallida* (Skrjabin & Schulz, 1937). In his revision of the Anoplocephalidae tapeworms, Spassky (1951) approved this division and concluded that the above-named species were valid. Although at least 12 *Moniezia* species have been reported in domestic and wild ruminants, it is difficult to distinguish them at the species level based on morphology alone. Among these, *M. expansa* and *M. benedeni* are the most widespread and harmful species (Schmidt, 1986). Currently, morphological classification of *Moniezia* is mainly based on the size of adult worms and the morphology of the scolex, interproglottidal glands, reproductive organs and eggs (Machida *et al.*, 1974; Liu *et al.*, 2008; Wang *et al.*, 2010b).

Using pathogens to detect monieziasis relies on the identification of eggs and/or a gravid proglottid. However, the shape of eggs is similar among closely

Table 2. Multiple sequence alignment of ITS1–5.8S fragment amplified from the 12 *M. sichuanensis* specimens.

Specimen ID	27	105	127	185	186	187	246	359	411	427	428	429	454	528	637	673
MEK01	G	T	G	G	G	T	G	T	A	A	T	C	T	T	A	T
MEK02	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
MEK03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BX01	C	.	A	–	–	–	.	.	.	.	.	.	.	.	.	.
BX02	C	.	A	–	–	–	.	.	.	.	.	.	.	.	.	.
BX03	C	.	A	.	.	.	.	C	.	–	–	–	.	.	.	.
BX04	C	.	A	–	–	–	.	.	.	.	.	.	.	.	.	.
FX01	C	.	A	–	–	–	.	.	G	.	.	.	C	.	G	.
FX02	C	.	A	–	–	–	.	.	G	.	.	.	C	.	G	.
LB01	C	C	A	–	–	–	.	.	G	.	.	.	C	C	.	C
LB02	C	C	A	–	–	–	.	.	G	.	.	.	C	C	.	C
ZP01	C	.	A	–	–	–	A	.	G	.	.	.	C	.	.	.

Specimen ID and nucleotide positions of point mutations are shown. 5.8S starts from nucleotide 633 in MEK01. Only variable positions are shown: '.' indicates that the nucleotide position corresponds to the MEK01 reference isolate, and '–' represents an alignment gap.

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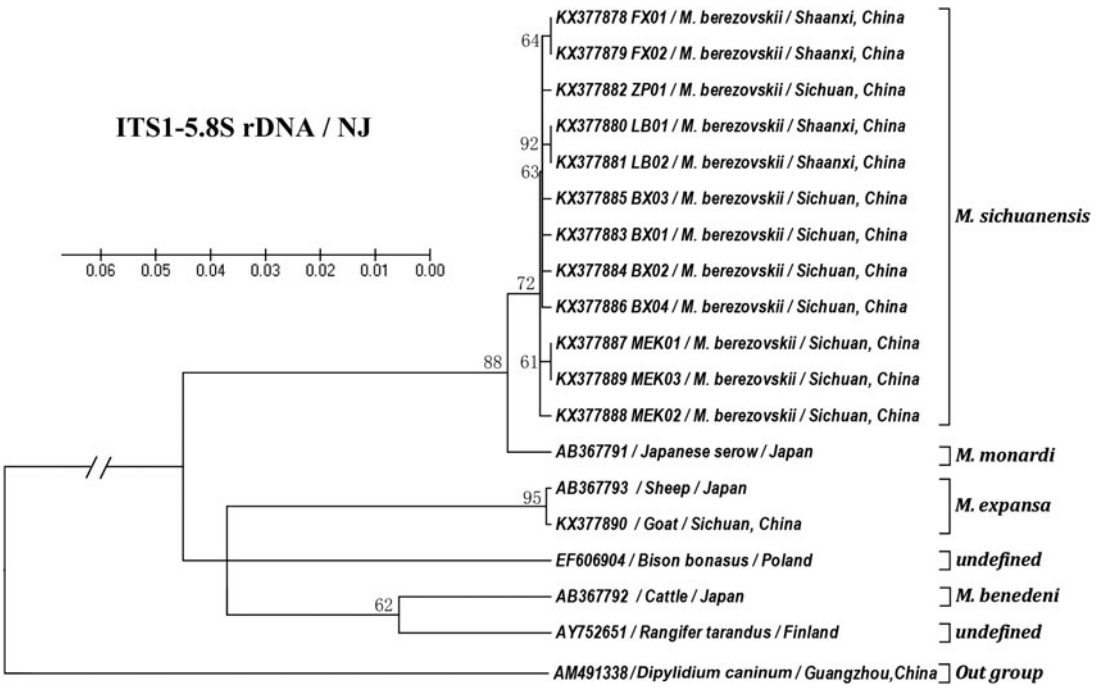

Fig. 3. Phylogenetic relationships between cestodes isolated from musk deer and other species of the genus *Moniezia*. Phylogeny was inferred based on the amplified ITS1–5.8S fragment using the neighbour-joining method. Numbers on nodes indicate bootstrap values resulting from phylogenetic analysis, and values less than 50% are not shown.

related *Moniezia* species. In addition, excreted eggs are sometimes deformed, preventing morphological differentiation at the species level (Taylor, 1967; Chilton *et al.*, 2007). Identification via the proglottids is difficult because some individuals lack interproglottidal glands, and the proglottids excreted in the faeces sometimes lack interproglottidal glands.

In recent years, molecular methods have been introduced that help the classification and determination of *Moniezia* spp., with cytochrome *c* oxidase subunit 1 (COX1) and ITS genes being the most frequently used markers (Nguyen *et al.*, 2012; Diop *et al.*, 2015; Ohtori *et al.*, 2015). ITS could serve as a good genetic marker because it can differentiate *M. expansa* and *M. benedeni* recovered from cattle, goats and sheep (Nguyen *et al.*, 2012). In addition, ITS can discriminate *M. monardi* from Japanese serows, *M. benedeni* from cattle and *M. expansa* from sheep (Ohtori *et al.*, 2015). Due to sequence variation caused by the rapid evolution of ITS genes, this gene could contribute to our understanding of the intra- and interspecific phylogenetic relationships among *Moniezia* spp., as well as the identification of a new species at the molecular level (Bowles *et al.*, 1995; Fazaeli *et al.*, 2000).

In the present study, the ITS1–5.8S sequences obtained from the 12 *M. sichuanensis* specimens showed some nucleotide variation. However, the similarity ranged from 98.7 to 100%, and between 67.8 and 92.4% when compared with other *Moniezia* spp. The phylogenetic trees showed that all 12 samples were closely related to each other and clustered together in the same strain. *Moniezia monardi*, sharing a similarity of 92.4%, was the species most closely related to *M. sichuanensis*. However, the original description of this species was based on material found in a reedbuck in Angola (the original paper is not available), while the genetic information was reported from Japanese serow (*Capricornis crispus*) (Machida *et al.*, 1974; Ohtori *et al.*, 2015). It is worth noting that the main feature that distinguishes *Moniezia* spp. is the interproglottidal glands. The interproglottidal glands of *M. expansa* and *M. monardi* are grouped around depressions that open to the surface of the segment (rosette type) (Machida *et al.*, 1974; Liu *et al.*, 2008; Wang *et al.*, 2010b; Ohtori *et al.*, 2015). However, genetic analysis showed that these two species are in different branches (75% identity), indicating a distant relationship (fig. 3). This is consistent with the report of Ohtori *et al.* (2015). Although a relatively high sequence similarity was observed between *M. monardi* and *M. sichuanensis* (92.4%), this variation exceeded the intraspecies variation (98.7–100%) within the *M. sichuanensis* group. Additionally, due to the shape of the interproglottidal glands (rosette-shaped vs. sawtooth-shaped), *M. monardi* and *M. sichuanensis* were thought to be different species belonging to different subgenera of the genus *Moniezia*.

In summary, this study reports the first molecular information on *M. sichuanensis*, thereby improving our understanding of the taxonomy and phylogenetic relationships of species in the genus *Moniezia*. Moreover, the combined morphological and molecular information

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on *M. sichuanensis* could facilitate the future identification of *Moniezia* spp. and the control of monieziasis.

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Conflict of interest

None.

Ethical standards

All procedures were reviewed and approved by the Animal Welfare Committee of China. During faecal collection, animal welfare was ensured throughout the sampling process.

Authors' contributions

J.X. and H.L. participated in the design of the study, performed all experiments, collected and analysed the data and wrote the manuscript; J.C., W.F., Y.C. and H.W. helped in collecting the faecal samples; X.G., W.L. and X.P. participated in the processing of specimens; G.Y. devised the study, participated in its design and coordination, helped to interpret the results and edited the manuscript. All authors read and approved the final manuscript.

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